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6. AUTHORS

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5d. PROJECT NUMBER

5e. TASK NUMBER

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14. ABSTRACT

The immediate scientific objectives, which have changed significantly from our original proposal due to overlap with a pre-existing ONR YIP Award, include:

(1) To engineer metabolic enzymes into functional multi-protein assemblies. We have explored the use of eukaryotic signaling scaffolds for in vivo enzyme assembly. [Note: the original proposal focused on using TGase-mediated enzymatic cross-linking to accomplish enzyme assembly]. The efficacy of these channels will be demonstrated for efficient metabolic

15. SUBJECT TERMS

propanediol biosynthesis, engineered bacteria, metabolic channels, enzyme compartmentalization

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a. REPORT	b. ABSTRACT	c. THIS PAGE			Matthew DeLisa
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Report Title

Final Report: A hybrid computational-experimental framework for microbial chemical synthesis via enzyme channelling

ABSTRACT

The immediate scientific objectives, which have changed significantly from our original proposal due to overlap with a pre-existing ONR YIP Award, include:

(1) To engineer metabolic enzymes into functional multi-protein assemblies. We have explored the use of eukaryotic signaling scaffolds for in vivo enzyme assembly. [Note: the original proposal focused on using TGase-mediated enzymatic cross-linking to accomplish enzyme assembly]. The efficacy of these channels will be demonstrated for efficient metabolic conversion of renewable resources (e.g., glycerol) to 1,2-propanediol.
(2) Enable combinatorial channel engineering via intracellular metabolite sensors. We have engineered a protein conformational switch based on the green fluorescent protein [note: that the original proposal sought to develop RNA aptamer-based switches] that we expect will dynamically respond to a broad concentration range of specific metabolites including R-1,2-PD.
(3) Computational design of optimal metabolic systems. We have shown through simulation that our synthetic channels locally improve the catalytic efficiency of the 1,2-propanediol enzyme assembly compared to the unchanneled case (Conrado et al., 2007 Metab Eng). To design optimal precursor flux to the 1,2-PD channel, we will develop new network design tools that can be used to computationally develop metabolic architectures that take full advantage of engineered assemblies.

List of papers submitted or published that acknowledge ARO support during this reporting period. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Conrado, R.J., Mansell, T.J., Varner, J.D. and DeLisa, M.P. (2007) Stochastic reaction-diffusion simulation of enzyme compartmentalization reveals improved catalytic efficiency for an engineered bacterial propanediol pathway. Metab Eng 9: 355-63.

Contreras Martinez, L., Borrero, E.E., Escobedo, F. and DeLisa, M.P. (2007) In silico protein fragmentation reveals the importance of critical nuclei in domain reassembly. Biophys J (in press).

Mansell, T.J., Fisher, A.C. and DeLisa, M.P. (2007) Engineering the protein folding landscape in Gram-negative bacteria. Curr Protein Pept Sci (in press).

Waraho, D. and DeLisa, M.P. (2007) A versatile selection technology for intracellular protein-protein interactions mediated by a unique bacterial hitchhiker transport mechanism. Proc Natl Acad Sci USA (in revision).

Number of Papers published in peer-reviewed journals: 4.00

(b) Papers published in non-peer-reviewed journals or in conference proceedings (N/A for none)

Conrado, R.J., Mansell, T.J. and DeLisa, M.P. (2007) Engineering multifunctional enzyme systems for optimized metabolite transfer between sequential conversion steps. In: Smolke, C.D., editor. Handbook for Metabolic Pathway Engineering. San Diego: CRC Press.

Number of Papers published in non peer-reviewed journals: 1.00

(c) Presentations

Conrado, R.J. and DeLisa, M.P. Intracellular enzyme crosslinking: towards multifunctional enzyme machines for the production of R-1,2-propanediol. 234th ACS National Meeting, August 2007.

Waraho, D. and DeLisa, M.P. A highly efficient selection strategy for intracellular protein-protein interactions mediated by a unique bacterial hitchhiker transport mechanism. 234th ACS National Meeting, August 2007.

Conrado, R.J. and DeLisa, M.P. Intracellular enzyme crosslinking: towards multifunctional enzyme machines for the production of R-1,2-propanediol. ECI Biochemical Engineering XV, July 2007.

Number of Presentations: 3.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts): 0

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts): 0

(d) Manuscripts

Number of Manuscripts: 0.00

Number of Inventions:

Graduate Students

NAME	PERCENT_SUPPORTED
Tom Mansell	0.00
Rob Conrado	0.00
Didi Waraho	0.00
FTE Equivalent:	**0.00**
Total Number:	**3**

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Faculty Supported

NAME	PERCENT_SUPPORTED	National Academy Member
Matthew DeLisa	0.00	No
Jeffrey Varner	0.00	No
FTE Equivalent:	**0.00**	
Total Number:	**2**	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED
Catherine Manix	0.00
Parbir Grewal	0.00
FTE Equivalent:	**0.00**
Total Number:	**2**

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period:	0.00
The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:.....	0.00
The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:.....	1.00
Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):	1.00
Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:	0.00
The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense	0.00
The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields:	0.00

Names of Personnel receiving masters degrees

NAME

Total Number:

Names of personnel receiving PHDs

NAME

Total Number:

Names of other research staff

NAME PERCENT_SUPPORTED

FTE Equivalent:

Total Number:

Sub Contractors (DD882)

Inventions (DD882)

Project Title: A hybrid computational-experimental framework for microbial chemical synthesis via enzyme channeling

A. Statement of the problem studied.

The *long-term objective* of our proposed studies is the development of a broad-spectrum platform for engineering metabolic channels in bacteria that is expected to dramatically improve the yield of virtually any biosynthetic target produced via metabolic engineering by accounting for enzyme organization and minimizing metabolic cross-talk. As proof-of-concept, we have chosen as a model system the microbial synthesis of 1,2-propanediol (1,2-PD) from renewable glycerol as feedstock.

Our approach to engineering microogranisms is significant because the production of high-value biobased chemicals by fermentation is gaining increased importance as nonrenewable resources are depleted and the world population grows. Microbial fermentation processes are particularly attractive as they use renewable feedstocks such as glucose or sucrose and do not generate toxic byproducts. The product to be investigated in this proposal, 1,2-PD, is a major commodity chemical with over 1 billion pounds produced annually in the U.S. and sells for about $0.81 per pound with a 4% growth in the market size annually. Some typical uses of 1,2-PD are in unsaturated polyester resins, functional fluids (antifreeze, de-icing, and heat transfer) transfer), pharmaceuticals, foods, cosmetics, liquid detergents, tobacco humectants, flavors and fragrances, personal care, paints and animal feed. In addition, 1,2-PD is a precursor to 1,2-PG dinitrate (PGDN) which comprises ~80% of the propellant used in torpedoes. The commercial route to produce 1,2-PD is by the hydration of propylene oxide derived from nonrenewable propylene by either the chlorohydrin process or the hydroperoxide process. Unfortunately, this chemical process has a number of disadvantages, including: (1) the use of a nonrenewable petrochemical derivative as feedstock, namely propylene; (2) the fact that several toxic chemicals, such as chlorine, propylene oxide, and propylene chlorohydrin are either required or are produced as by-products; and (3) the use of large quantities of water to minimize the production of polyglycols. Thus, the ability to produce 1,2-PD via microbial fermentation has the potential to displace a nonrenewable petroleum-based feedstock and impact the environment by reducing both the use and production of toxic byproducts and the consumption of water. Finally, it should be reiterated that the technology developed in this proposal is envisioned to be generic for a wide range of engineered metabolic pathways; thus similar energy and environmental impacts would be expected for any biobased compound produced using this strategy.

B. Summary of the most important results.

The most important scientific results, which have significantly changed from our original proposal, include:

(1) Demonstration of efficient multi-protein assembly in bacterial cells. We have explored the use of eukaryotic signaling scaffolds for in vivo enzyme assembly. [Note: the original proposal focused on using TGase-mediated enzymatic cross-linking to

accomplish enzyme assembly]. The efficacy of these channels will be demonstrated for efficient metabolic conversion of renewable resources (e.g., glycerol) to 1,2-propanediol. **(2) Creation of metabolite sensors for further engineering metabolic channels.** We have engineered a protein conformational switch based on the green fluorescent protein that we expect will dynamically respond to a broad concentration range of specific metabolites including R-1,2-PD. Note that this is a departure from the original proposal as we have learned from experimentation that RNA-based switches are not going to be feasible for detecting small molecules that lack steric bulk.

(3) Computational design of optimal metabolic systems. We have shown through simulation that our synthetic channels locally improve the catalytic efficiency of the 1,2-propanediol enzyme assembly compared to the unchanneled case (Conrado et al., 2007 Metab Eng). To design optimal precursor flux to the 1,2-PD channel, we will develop new network design tools that can be used to computationally develop metabolic architectures that take full advantage of engineered assemblies.

The specific major accomplishments to date include:

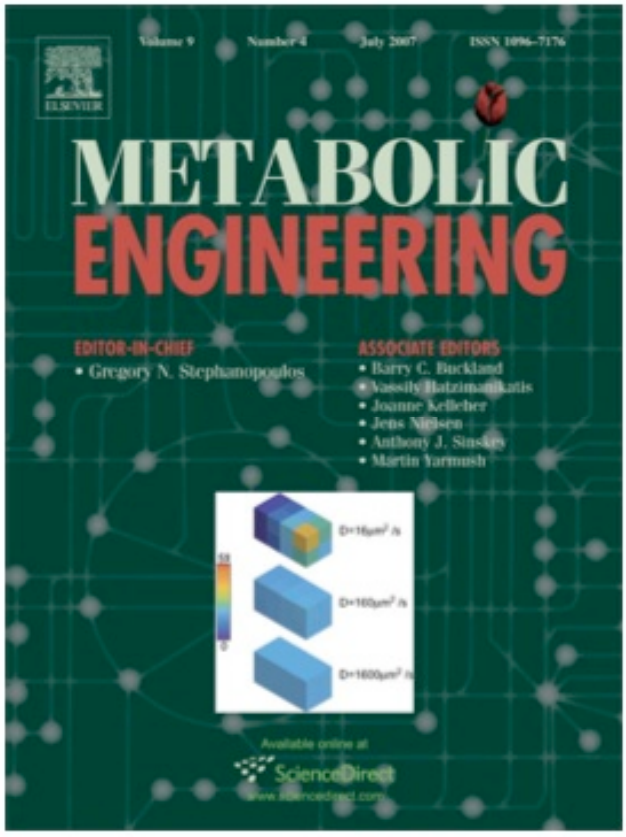
(1) We have tested all possible 2- and 3-way enzyme fusion proteins, exploring factors such as spacing between enzymes (i.e., linker length), expression level (i.e., promoter strength, inducer level) and alternative pathway enzymes (i.e., homologues from different species). Remarkably, we have discovered that direct translational fusion between two *R*-1,2-PD pathway enzymes, namely methylglyoxal synthase (MGS) and glycerol dehydrogenase (GldA) results in a nearly 8-fold improvement in *R*-1,2-PD titers compared to cells expressing unfused MGS and GldA (0.895 g/L versus 0.11 g/L, respectively). This is significant as it clearly demonstrates that co-localization of metabolic pathway enzymes can have a profound impact on metabolite production. In parallel, we have also begun to scaffold enzymes using components or modules from eukaryotic signaling systems. For instance, by fusing our metabolic enzymes to interacting domains borrowed from eukaryotic signaling proteins, we have been able to direct the non-covalent assembly of two or more enzymes in the cell. We are now evaluating the impact of this assembly on 1,2-PD titers.

(2) Our second major accomplishment has been the development of a protein conformational switch, based on the green fluorescent protein (GFP), that upon binding to small molecules of interest is dramatically stabilized *in vivo*, resulting in a large increase in measurable cell fluorescence. This was accomplished by generating a fusion protein between GFP and the TraR transcriptional activator from *Agrobacterium tumefaciens*. In the absence of its natural ligand, the freely diffusible quorum signaling molecule 3-oxooctanyl-l-homoserine lactone (OHHL), the TraR protein is a monomer that is highly unstable in the cytoplasm of *A. tumefaciens* and *E. coli*. However, upon biding of OHHL, TraR forms an extremely stable dimmer. We have observed that this same OHHL-dependent stability can be extended to an engineered GFP-TraR fusion protein. That is, in the absence of OHHL, GFP-TraR is highly unstable and cells expressing the fusion are relatively non-fluorescent. However, upon addition of OHHL, the GFP-TraR protein is stabilized (presumably in a dimeric conformation) and the cells

become highly fluorescent. We are now exploring the further engineering of this GFP-TraR conformational switch for sensing molecules other than OHHL. We expect that a bevy of small molecule switches can created using the GFP-TraR backbone, simply by the application of directed evolution to change the substrate specificity of TraR from OHHL to virtually any compound of interest.

(3) Our third major accomplishment has been the demonstration that enzyme co-localization has a dramatic effect on the titers of *R*-1,2-PD that can be achieved in *E. coli.* This was first demonstrated via computer simulation in collaboration with Dr. Jeffrey Varner (co-PI; Cornell University, School of Chemical and Biomolecular Engineering). Specifically, we developed a spatial stochastic model of *E. coli* central carbon metabolism using the Next Subvolume Method, an efficient implementation of the Gillespie direct method of stochastic simulation. Using this model, we demonstrated that synthetic channels *locally* improve the catalytic efficiency of the 1,2-PD enzyme assembly compared to the un-channeled case (Conrado et al. 2007). This work was featured on the cover of the July issue of the journal *Metabolic Engineering* (see below).

Conrado RJ, Mansell TJ, Varner JD and DeLisa MP (2007) Stochastic reaction-diffusion simulation of enzyme compartmentalization reveals improved catalytic efficiency for a synthetic metabolic pathway. *Metab Eng* **9:** 355-63



C. Technology transfer.

In terms of technology transfer, we are in the very early stages of developing relationships with industrial partners that are interested in the technologies developed under this award. In the last year, we have filed or will file 2 patents on technologies that resulted from ARO-funded research. These include the following:

1. Compositions and methods for analyzing protein interactions (PCT/US2006/032810)

2. Genetic methods for optimizing metabolic pathways (To be disclosed shortly)

The first technology listed above has been licensed exclusively to a biotechnology company called Vybion, Inc (www.vybion.com). In addition, a Cooperative R&D Agreement has been created with Merck & Co., Inc. to explore these protein folding and interaction technologies for the development of protein therapeutics. In the future, we expect a spin-off company to form based on the technology covered in the latter patent entitled "Genetic methods for optimizing metabolic pathways". The focus of this company (Akiva, LLC) will be to develop hybrid computational-experimental frameworks for high-value metabolite production in bacteria via enzyme channeling. The co-founder of this company, Dr. Jeffrey Varner (Cornell University), is an expert in large-scale mathematical models, has numerous years of industrial experience at Genencor International, Inc.